

**BEHAVIOR STUDY ON RATS WITH UNILATERAL SPLINT: IS SUDDEN
CHANGE IN OCCLUSION RESPONSIBLE FOR TMD PAIN?**

by

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University of Pittsburgh, 2018

Temporomandibular Joint (TMJ) Disorders (TMDs) affect 5-10% of the US population. One potential cause of TMD is a change in occlusion from trauma or surgery. In order to investigate whether a sudden change in occlusion is associated with the emergence of hypersensitivity in the TMJ area in adult rats, we performed perioral hypersensitivity assessment before and after splint placement on 12 male and 16 female Sprague Dawley rats with the orofacial pain assay. Rats were trained to access sucrose solution via a window in the side of the cage. Cumulative contact time (CT) with the sucrose sipper tube was determined for each 10 min training and subsequent testing session. For testing, 18-pin wire arrays were placed in the window to provide bi-lateral mechanical stimulation of the face when the sucrose solution was accessed. Baseline CTs were collected 3 times before the splint, and post-splint CTs were collected on different days depending on different batches. Splints consisted of dental resin poured to about 1 mm in thickness, which were applied unilaterally to the left maxillary molars. The experiment was conducted through 4 batches with 4 rats in the first batch and 8 rats in the other 3 batches. For the result, some of the rats in the splint group showed a decreasing trend for CT after splint placement while the others showed a transient decrease. For the Sham group, most rats in Batch 1 and 3 had very low CTs, while Batch 2 had high CTs before the splint but they decreased a lot after the splint placement. Batch 4 had high and stable CTs. No conclusion can be drawn from the current study because low CTs were seen in

many rats before splint placement. For the future studies, more training could increase the baseline CT in order to detect the impact of splinting

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1.0 Introduction

The Temporomandibular Joint (TMJ) is a bilateral synovial joint that articulates the condyle of the mandibular bone with the glenoid fossa of the temporal bone. A fibrocartilaginous intra-articular disc separates the two bones, providing lubrication and cushion for the joint. (Gallo, Nickel et al. 2000, Beek, Koolstra et al. 2001). Translation and rotation are two distinct movements involved in the opening and closing of the mandibular bone, but condylar pathways and the position of the disc can vary depending on the position of the condyle to the fossa.

Due to the structural and kinematic complexity of the TMJ, TMJ Disorders (TMDs) are not uncommon. According to the National Institute of Dental and Craniofacial Research (NIDCR), 5~12% of the US population is affected by TMDs (NIDCR , Von Korff, Le Resche et al. 1993). TMDs are reported to cause severe disability and negatively affect patients' quality of life (Dahlstrom and Carlsson 2010, Velly and Friction 2011).

TMD involves the TMJ and/or the surrounding muscles, leading to clicking or grating sounds, joint or facial pain, decreased jaw movement, and jaw popping or locking, depending on the severity of the disease. For most of the TMD patients, these symptoms can disappear by themselves without any treatment, but for a small portion of the patients, the symptoms can persist and develop into persistent pain and joint dysfunction, which contributes about 85% of the cost for treating TMD among all the patients (Friction and Schiffman 1995).

It is estimated that TMDs are more prevalent in females than in males, and the female to male ratio is somewhere from 2:1 to 8:1 (NIDCR , Shaefer, Holland et al. 2013, Bueno, Pereira et al. 2018). Hormones are believed to play an important role in the sexual difference of prevalence. According to LeResche et al. and Smith et al., women will experience higher levels of TMD pain

during menstruation and ovulation, which are characterized by rapid decrease and fluctuation of estradiol (LeResche, Mancini et al. 2003, Smith, Stohler et al. 2006).

Although the etiology of TMDs is still speculative, three main contributing factors have been proposed from previous studies: anatomical factors (e.g. trauma, change in occlusion), pathophysiological factors (e.g. the difference in hormone, or peripheral sensation), and psychosocial factors (e.g. stress, anxiety, depression) (Wadhwa and Kapila 2008, Maixner, Diatchenko et al. 2011). Tanaka et al. propose that the degeneration of the TMJ cartilage starts with the mechanical loading exceeding the adaptive capacity of the joint, because of the increased mechanical factors (trauma, change in occlusion) and/or reduced mechanical capacity of the joint (hormone alteration, emotional changes) (Tanaka, Detamore et al. 2008). The mandibular bone is connected to the skull mainly through TMJs and the occlusion, so the stability of the occlusion will affect the stability of the two joints. During normal mastication, the condyle will rotate and slide anterior-inferiorly out of the glenoid fossa to the articular tubercle of the temporal bone, squeezing the TMJ disc equally on both sides. When the normal occlusion is disturbed by trauma or an unbalanced denture, erratic movement of the joint will occur, resulting in shifted load between the two joints. Such shifting can cause decreased loading on one joint and excessive loading on the other, which could be a predisposing factor to TMJ degeneration.

A change in occlusion is very different to classically defined malocclusion. In a recently published review paper, the causal relationship between malocclusion and TMD was concluded after the in-depth study of 52 clinical studies. The authors attributed the controversial conclusion to the lack of precise definition and classification of malocclusion and TMD (de Kanter, Battistuzzi et al. 2018). However, according to another review paper, which includes 25 studies, the majority (23) came to the same conclusion that there is no association between malocclusion and TMD, and

even for the two that shows the association, no causal relation can be concluded (Manfredini, Lombardo et al. 2017).

The pathological change of TMJ degeneration includes irreparable abrasion of articular fibrocartilage and thickening and remodeling of underlying bone (Zarb and Carlsson 1999). Our recent study using the rabbit unilateral splint TMD model showed an almost complete loss of the subchondral layer of the fibrocartilage accompanied by a loss of defined cell layer in the posterior regions of the contralateral condyle within a 6-week period. The mechanical test has also shown a change in mechanical properties of the fibrocartilage in the posterior region of the condyle due to the significant degeneration of the subchondral collagen type II/GAG rich layer (Henderson, Lowe et al. 2015). However, it is still not known whether such pathological change in the condyle is associated with the emergence of the TMD pain.

In order to address the questions mentioned above, the objectives of this study are: (1) to apply the Orofacial Pain Assay to the rats with unilateral splints to see if altered TMJ loading and TMD pain/facial hypersensitivity are related; (2) to determine whether gender difference will affect the development of TMD pain/facial hypersensitivity in rats. By conducting the study, we are hoping to provide the evidence of correcting the altered TMJ loading for the treatment of TMJ pain in the patients, and the guidance to surgeons on the window in which interventions to normalize loading may be most successful.

2.0 Hypothesis

The total Contact Time (CT) of splinted rats is lower than that of the Sham rats within 4 weeks after the splint placement.

3.0 Materials and Methods

3.1 Animals

12 male and 16 female Sprague Dawley rats were used in this study. They were divided into 4 batches: the first batch includes 4 male rats, the second batch includes 8 female rats, the third batch includes 8 male rats, and the fourth batch includes 8 female rats (Table 1). The rats were 6-8 weeks old by the time they arrived at the housing facility, Biomedical Science Tower 3 at the University of Pittsburgh. Two rats of the same gender were housed in one chamber, where they had free access to water and hard food pellet 24/7. The rats were settled for 72 hours before any handling. Rats were randomly assigned into Sham group and experimental group in Batch 2, 3, and 4. Batch 1 was a polite study, thus all 4 rats were served as the experimental group.

Batch	Gender	Number	Splint	Sham	Test Length	Sucrose Water	Re-habitation?
1	M	4	4	0	4 wks.	5%	N
2	F	8	4	4	4 wks.	30%	N
3	M	8	4	4	4 wks.	30%	Y
4	F	8	4	4	6 wks.	5%	N

Table 1 List of differences between batches

3.2 Orofacial Pain Assay

Orofacial Stimulation Test (Fehrenbacher, Henry, Hargreaves method) from Ugo Basile (Model#: 31300) was used in this study. It consists of an operative chamber where rats have access to a sipper tube with 5% (30% for Batch 2 and 3) sucrose solution (Figure 1A). To access the tube, the rats have to poke their nose through a hole in which mechanical stimuli (thin wires) make contact with the face (Figure 1B). The stimulus is normally innocuous, but rats will become aversive if there is hypersensitivity in the skin or underlying muscle tissue. The dependent measure is the Total Contact Time (CT) of sipper lick during a 10-minute session. Whenever the rats make contact with the sipper, breaking the infrared beam behind the hole, the beam sensor will record the duration of every contact and input it into the computer as the CT value. Two rats were tested simultaneously in two different operative chambers in the same room.

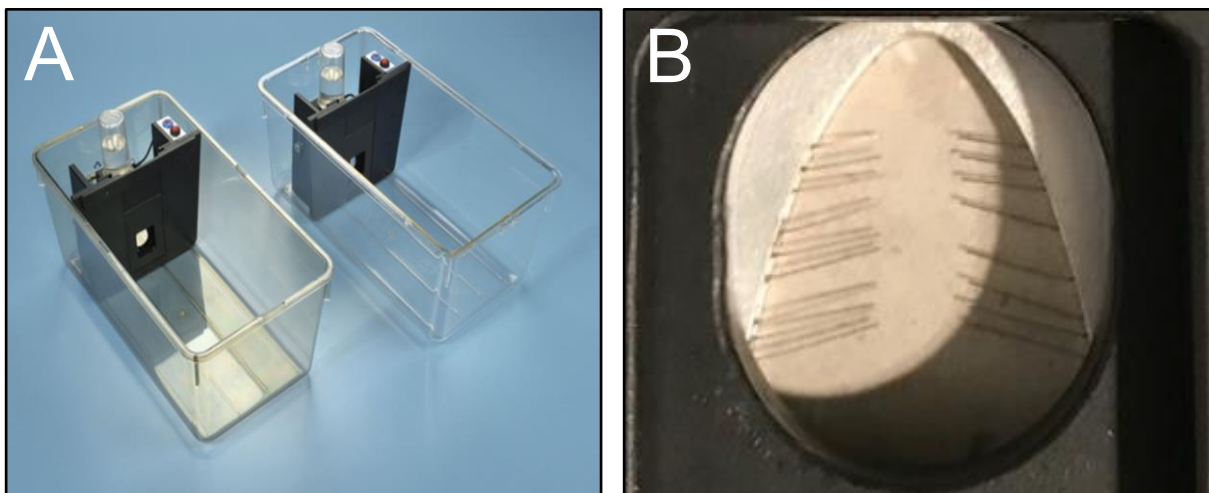


Figure 1 Orofacial Pain Assay

A. Operative chambers; B. 18-pin wire arrays.

3.2.1 Habituation & Baseline Data Collection

After the rats were settled for 72 hours, they were habituated to the nociceptive testing apparatus for 10 min per day for 5 consecutive days (4 weeks for Batch 2). During the habituation, no mechanical stimuli were applied, allowing them to have free access to the 5% (30% for Batch 2 and 3) sucrose solution.

After the 5-day habituation period (4 weeks for Batch 2), thin wires were attached to the hole, applying mechanical stimulation to the face of the rats when they access the sucrose solution. CT was collected as baseline data during a 10-minute session per day for 3 days.

3.2.2 Splint Placement & Checkup

All the rats were sedated with intraperitoneal Ketamine (0.55/kg), Xylazine (0.275 ml/kg), and Acepromazine (0.11 ml/Kg), and maintained in a surgical plane with a rack to open the mouth. 38% phosphoric acid etching gel was applied to the left maxillary molar and washed with water after 30 seconds. The teeth were then dried with the cotton swab, followed by primer application. Injectable resin of approximately 1mm thickness was applied and cured by UV-light onto the occlusal surface of the molar (Figure 2). For the Sham group, resin splints were removed right after the splint placement.

Oral checkups were performed after each test session to make sure all the splints were in place. Both the Sham and experimental group were anesthetized with 4% Isoflurane inhalation after the nociceptive testing. Missing splints were noted in 4 of the 16 rats in the splint group across all batches, which were re-splinted following the procedure described above.

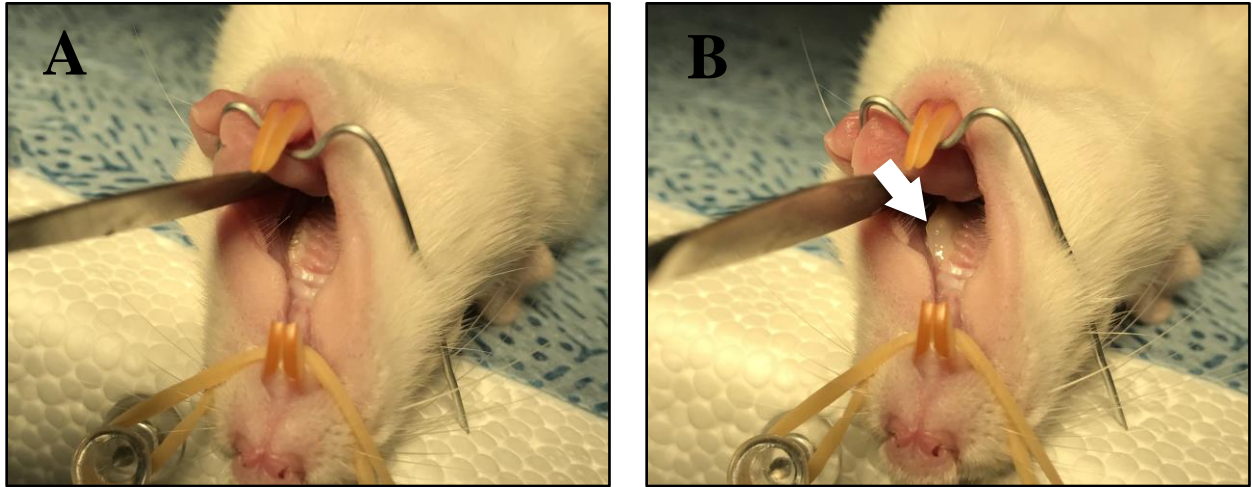


Figure 2 Surgical Placement of Splint

Splints were placed on the occlusal surface of left maxillary molar in all the rats: A. before; B. after. Note the resin splint indicated by the arrow.

3.2.3 Orofacial Pain Assay Post Splint Placement

For Batch 1, the nociceptive test was performed once per week after the splint placement for a total of 4 weeks, while for Batch 2, 3, the nociceptive test was performed daily during the first week and once per week thereafter for a total of 4 weeks. For Batch 4, the nociceptive test was performed three times for the first week and one per week thereafter for a total of 6 weeks.

18-pin wires were attached to the hole, applying mechanical stimulation to the face of the rats when they access the sucrose solution. Whenever the rats make contact with the sipper, breaking the infrared beam behind the hole, the beam sensor will record the duration of every contact and input it into the computer. The total Contact Time (CT) to the sipper during the 10-minute period was recorded and compared to the CT before the splint placement.

In Batch 3, a 10-minute session of re-habitation was performed every week one day before the actual test to boost the motivation for the rats. During the re-habitation, no wire was attached to the hole to allow free access to the sucrose water.

3.3 Weight Measurement

In order to evaluate how splint placement can affect the intake of food and thus the weight gain in the rats, their weight was measured and recorded after each test for Batch 3 and 4.

3.4 Statistical Analysis

All the CTs before and after the splint were compared in each rat through a two-tailed student t-test with $p < 0.05$ being statistically significant. Then the CTs of all the rats in the same group was averaged together, a two-tailed student t-test ($\alpha = 0.05$) was performed to compare the CTs before and after the splint placement in both the splint and Sham group, so as to evaluate the effect of splint placement on orofacial hypersensitivity.

4.0 Results

4.1 Orofacial Pain Assay

4.1.1 Male Rats Splinted for 4 Weeks - Pilot Study

Total Contact Time (CT) to the sipper during the 10-minute period was recorded as previously described using the orofacial pain assay apparatus. We included only 4 male rats as splint group without Sham group.

Figure 3 shows the CT changes in each rat. Among 4 rats, 2 showed a decrease trend post-splint as we previously expected. Despite partial fluctuation, Rat 1, 2, and 4 showed an overall decrease trend in CT after placing the splint. This trend is also seen where CTs are averaged for all the 4 rats (Figure 4). The only rat that did not show the same tendency had a low CT during the pre-splint period, which might prevent the CT from having a large decrease.

Note that there was a slight increase by the end of the 4-week testing period in Rat 2 and 4. This might due to a change in the environment because every rat showed the same pattern, or it could be due to the recovery of the degenerated condyle.

The CTs before and after the splint placement were compared on each rat, and only Rat 4 showed significant difference before and after the splint placement, while Rat 1 and 2 only showed a trend of decreasing CT after the splinting ($p < 0.05$, Figure 5). The reason why Rat 1 and 2 did not show a significant decrease might due to the fluctuation of the baseline data, and the slight increase at the end of the test. Also, the CTs during the pre-splint period is relatively low compared

to previous experience, which might cause a flooring effect on the post-splint data, resulting in a not significant outcome.

When combining the 4 rats, the CTs showed a significant decrease after splint placement ($p < 0.05$, $n = 4$, Figure 6). However, no conclusion can be drawn from this batch due to the lack of Sham group, but it showed the likelihood that a sudden change in occlusion might lead to TMD pain.

It is worth to mention that the decrease of CT in Rat 1 and 4 started within the first week after the splint placement, which implied the development of degeneration in the TMJ, and that TMD pain might happen within one week of the occlusal perturbation. So we introduced more tests in the first week post-splinting in subsequent batches to investigate the exact date of CT decrease.

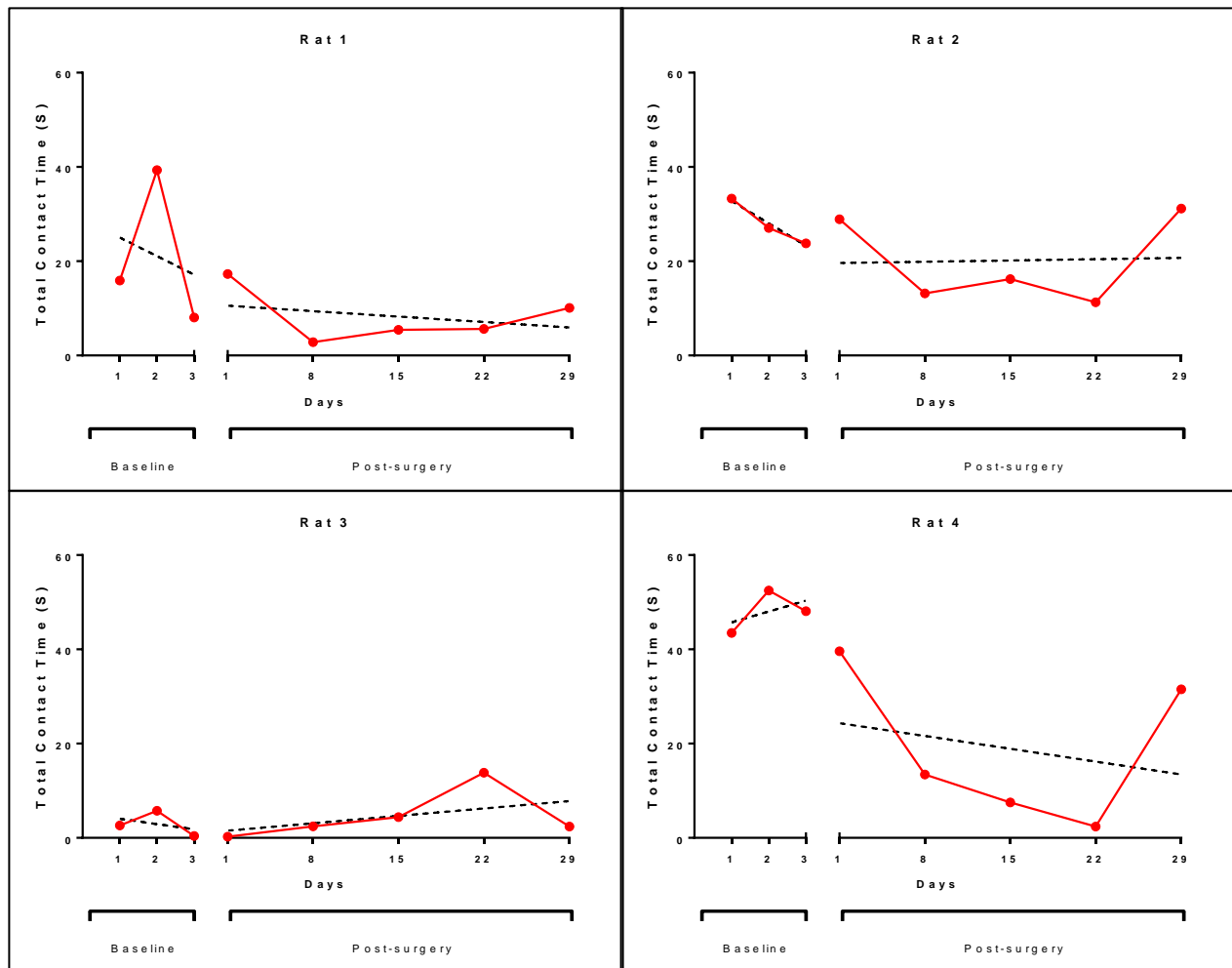


Figure 3 CT changes of each rat in Batch 1

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. Despite partial fluctuation, Rat 1 and 4 showed an overall decrease trend in CT after placing the splint. CT time of Rat 3 before the splint was relatively low, which might imply a flooring effect. Note that in Rat 2 and 4, there was a slight increase by the end of the 4-week testing period. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

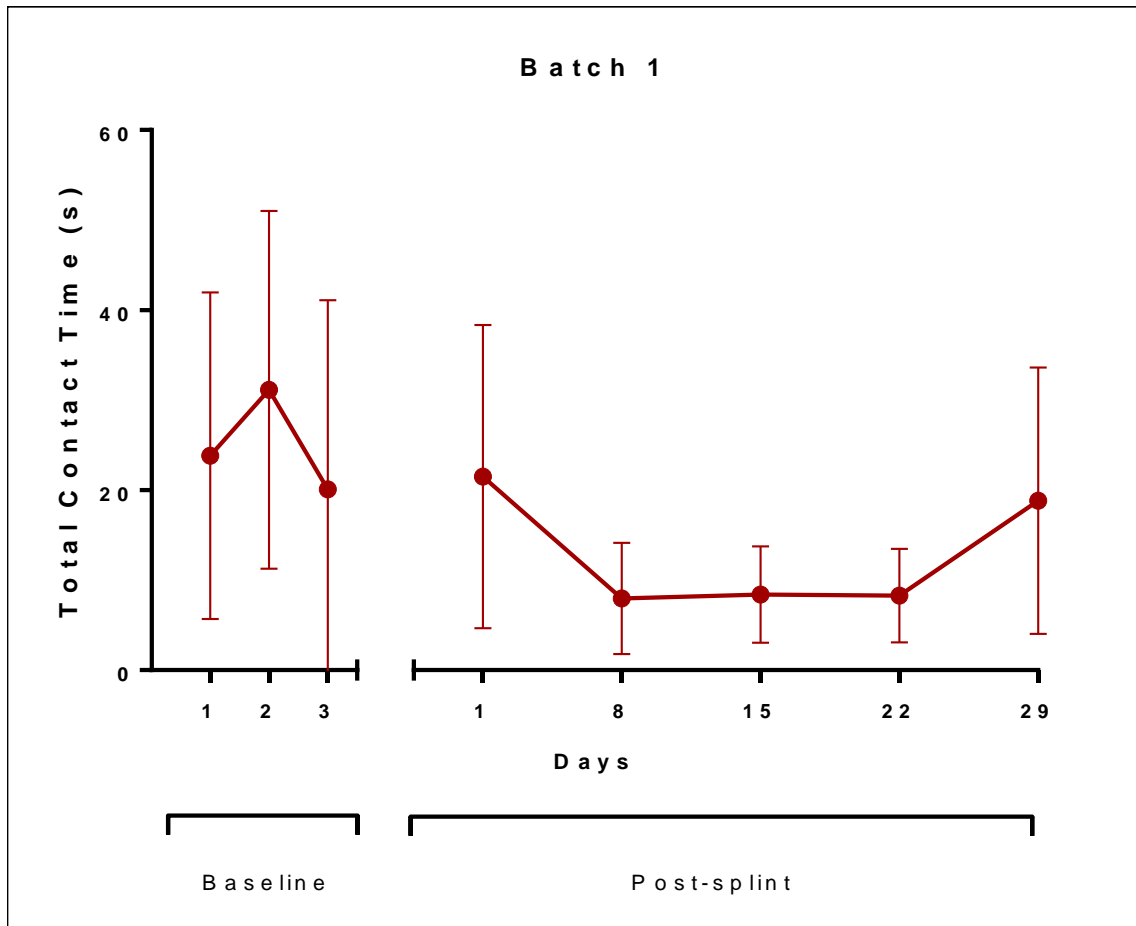


Figure 4 Averaged CT changes of all the rats in Batch 1

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. After averaging the CTs of all the 4 rats, the same decreasing trend as in Rat 1, 2, and 4 was seen post splint placement.

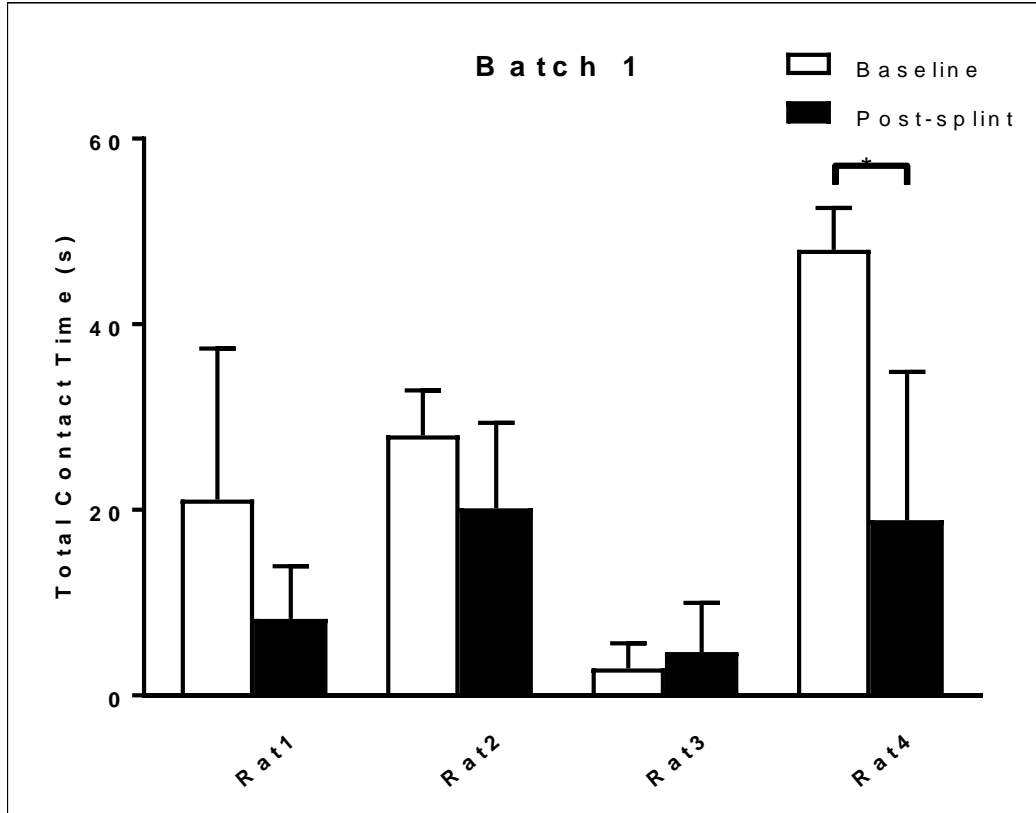


Figure 5 Averaged CT of each rat in Batch 1

The CTs before and after the splint were compared in each rat to evaluate the effect of splint placement on orofacial hypersensitivity. Although there was a decreasing trend in Rat 1 and 2, the significant difference was only seen in Rat 4. The asterisk indicates a statistical difference ($\alpha=0.05$).

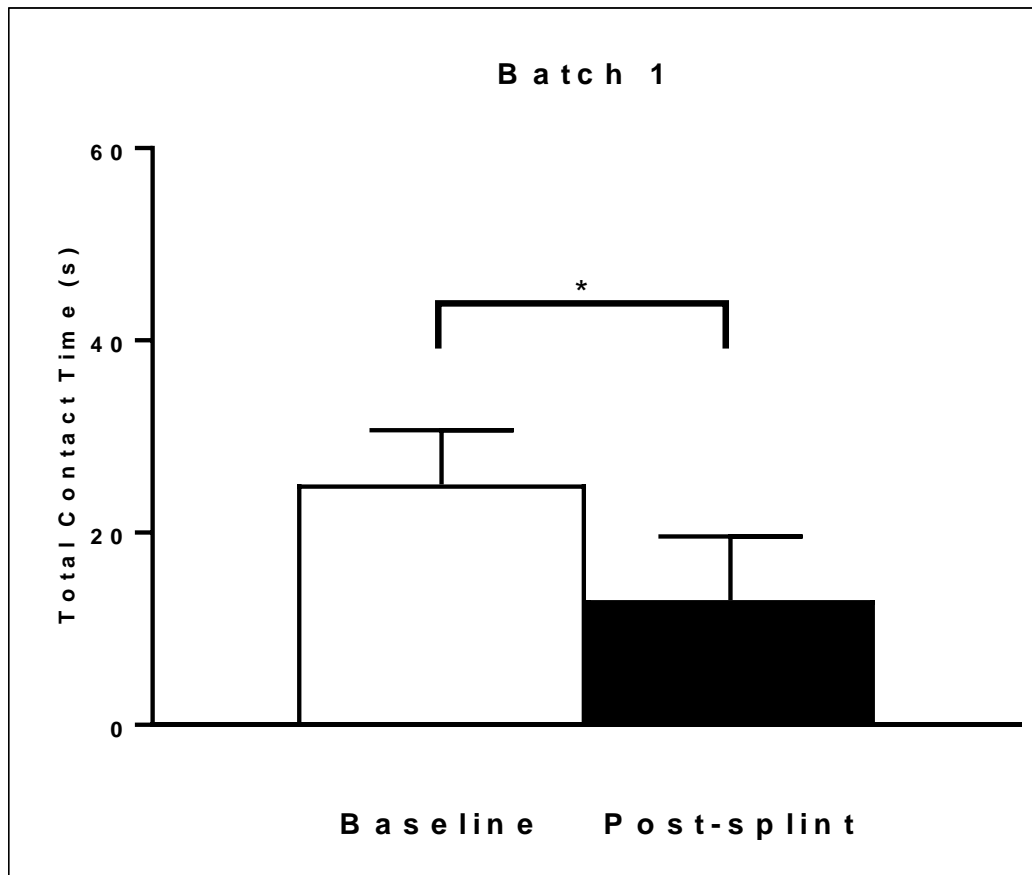


Figure 6 Averaged CT of all the rats in Batch 1

The CTs before and after the splint were compared in all the rats to evaluate the effect of splint placement on orofacial hypersensitivity. The graph showed that there is a significant difference before and after the splint placement. The asterisk indicates a statistical difference ($\alpha=0.05$).

4.1.2 Female Rats Splinted for 4 Weeks

Batch 2 consisted of 8 female rats, which were randomly and evenly distributed into the splint and Sham group. The CTs during the habituation period were very low (data not shown), so we increased the sucrose water concentration to 30% and extended the habituation period to 4 weeks. With those efforts, the baseline CTs reached around 100-200s, which was acceptable.

The line charts show the CT changes of each rat in the splint group (Figure 7) and the Sham group (Figure 8). The CTs of Rat 1 and 3 in the splint group showed an overall decrease despite some fluctuation during the first week after placing the splint, which is consistent with what we saw in Batch 1. CT of Rat 2 stayed at a high level for the first week before a dramatic drop in the last 3 weeks. The CT of Rat 4 experienced an increase, followed by a decrease during the first week, while Rat 3 fluctuated at a high level during the first week. For the Sham group, most of the rats showed a fluctuation for the first week.

After averaging the CTs of splint and Sham group, both groups show a similar trend – CT fluctuated at a high level for the first week followed by a sudden drop in the second week (Figure 9).

Besides Rat 2 in the Sham group, the averaged CTs for each rat did not show a significant difference before and after the splint in both groups ($\alpha=0.05$, Figure 10). And the combined CTs for the all the rats in both group also did not show a significant difference ($\alpha=0.05$, Figure 11).

During the first week, the wire pins were worn out gradually, which were fixed by attaching new wires to the wire pad, and the study was resumed without any re-habituation. After fixing the pads, the abrupt drop on day 14 indicated that the rats were affected by the change.

Due to the change of the experiment set up, it is pointless to compare the data recorded after the first week to data collected before. But we could still see the trend of decreasing CT in some of the splinted rats.

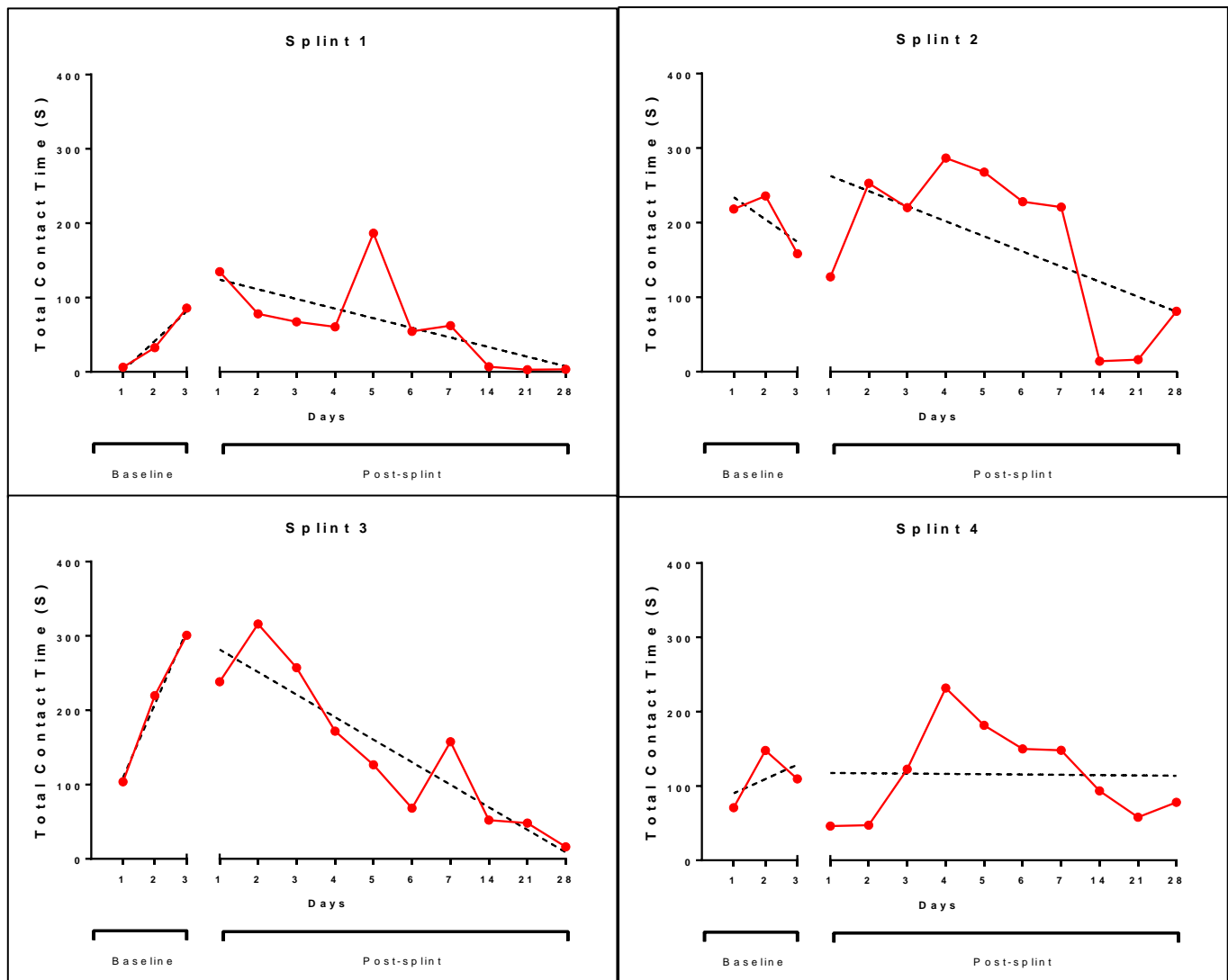


Figure 7 CT changes of each rat in the splint group in Batch 2

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. Rat 1 and 3 showed an overall decrease trend in CT after placing the splint despite some fluctuation. CT of Rat 2 stayed at a high level for the first week before a dramatic drop in the last 3 weeks. In Rat 4, CT increased for the first 4 days post-splint, followed by a continuous decrease. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

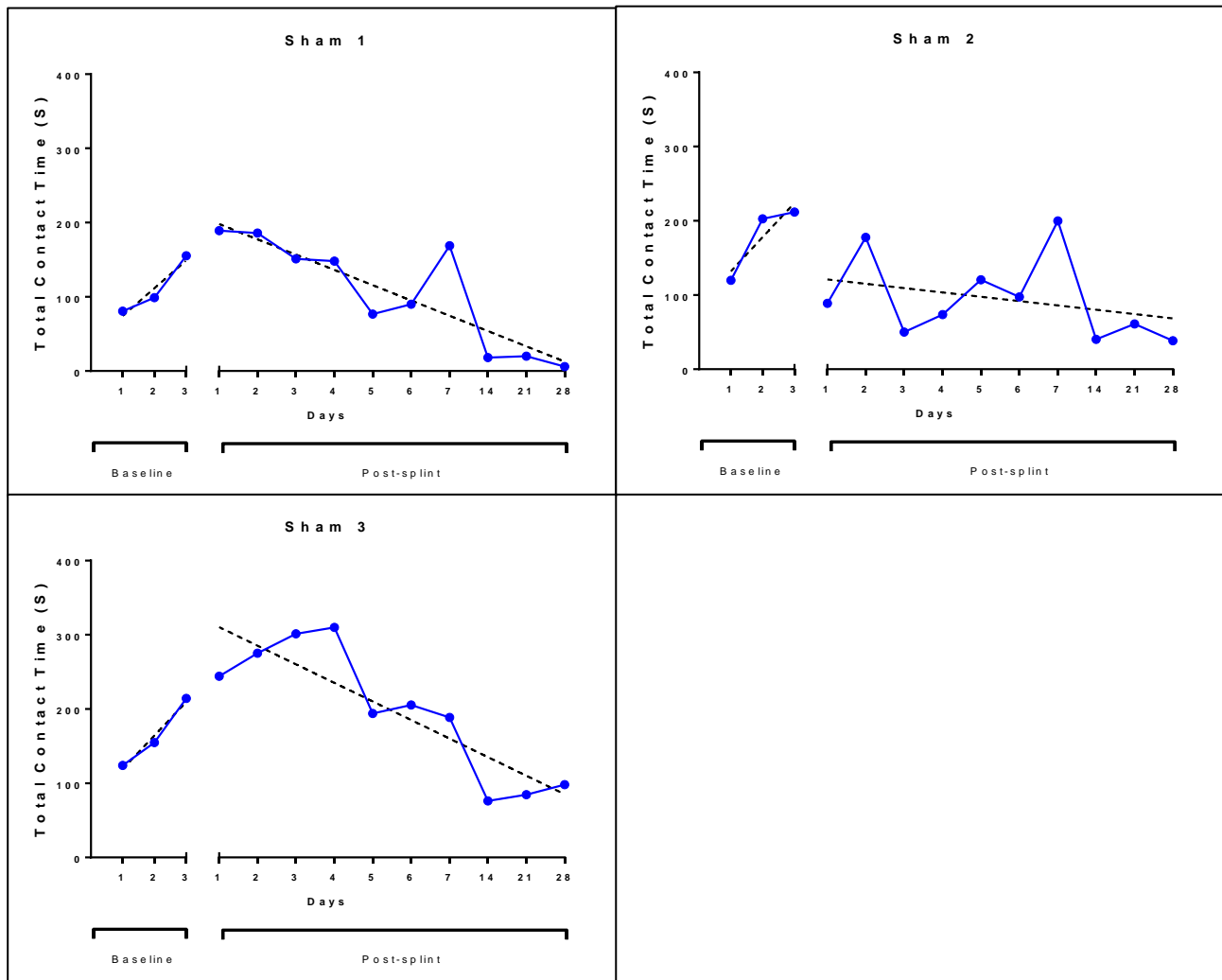


Figure 8 CT changes of each rat in the Sham group in Batch 2

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. Rat 3 showed a gradual decrease in CT, while Rat 1 and 2 exhibited fluctuated CTs followed by an abrupt drop starting the second week. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

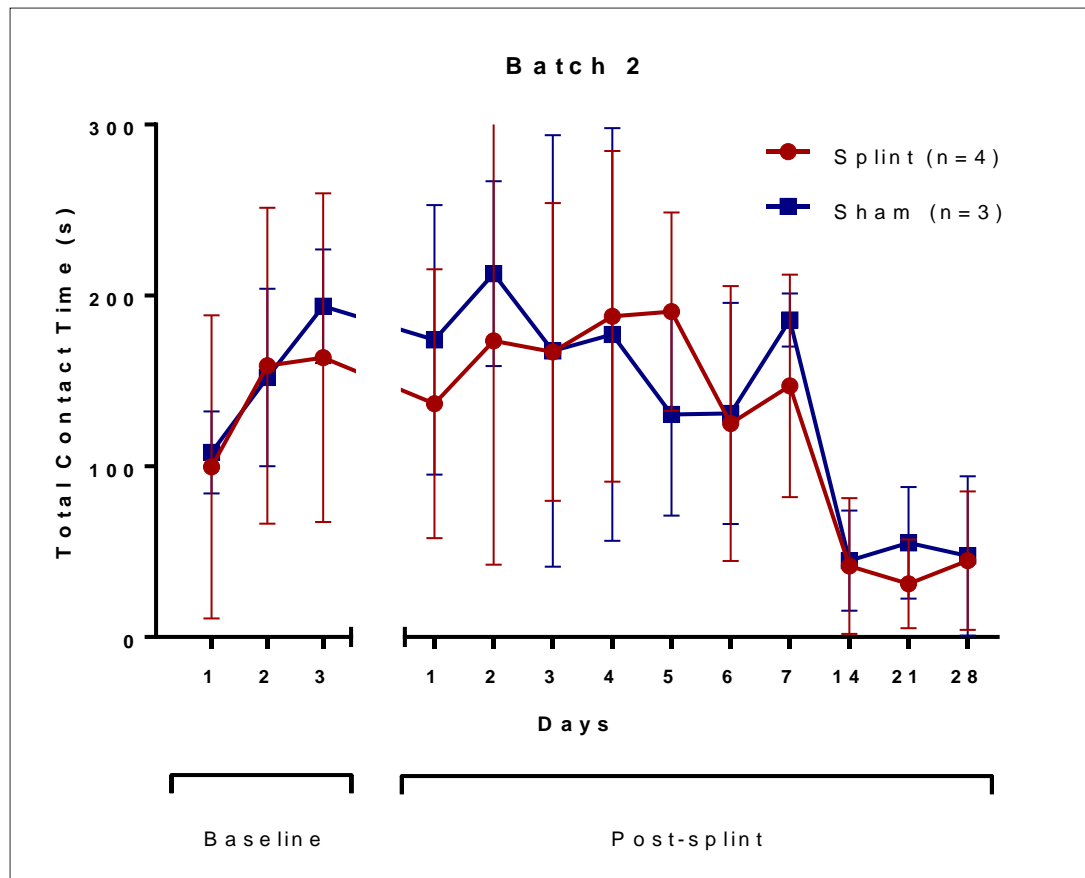


Figure 9 Averaged CT changes of all the rats in Batch 2

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. After averaging the CTs of all the 4 rats in the splint and Sham group, it is shown that the CT of both groups fluctuated at a high level for the first week followed by a sudden drop at the second week. The wire pads were broken down after the first week. After fixing the pads, the abrupt drop on day 14 indicated that the rats were affected by the change.

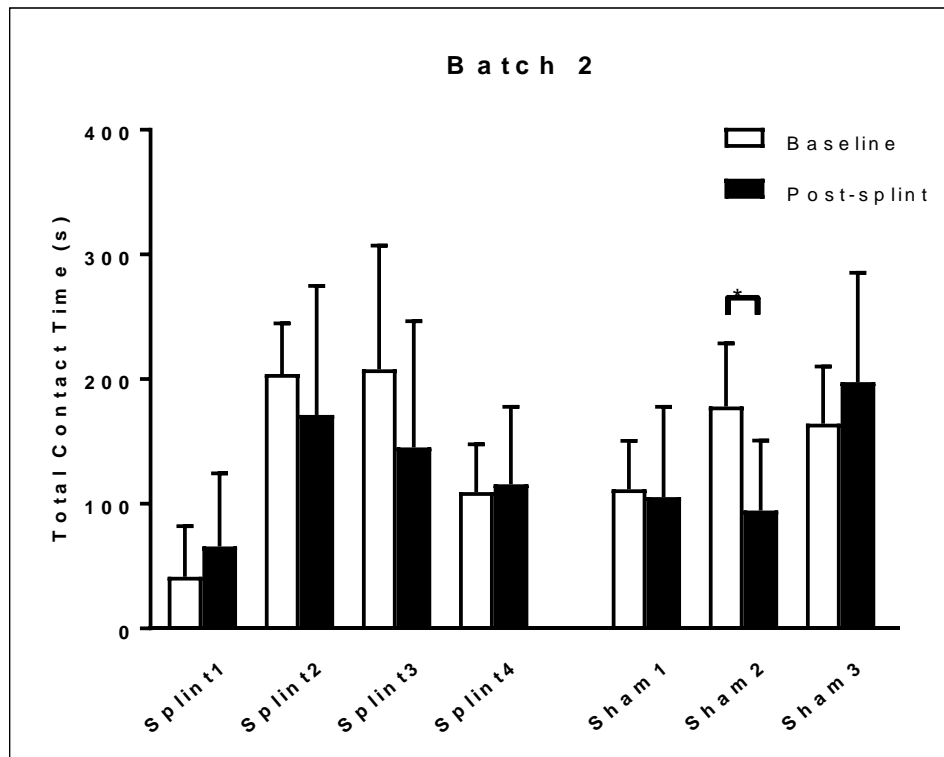


Figure 10 Averaged CT of each rat in Batch 2

The CTs before and after the splint were compared in each rat to evaluate the effect of splint placement on orofacial hypersensitivity. Rat 2 in the Sham group showed a significant difference. The averaged CTs for other rats, both in the splint and Sham group, did not show any significant difference. The asterisk indicates a statistical difference ($\alpha=0.05$).

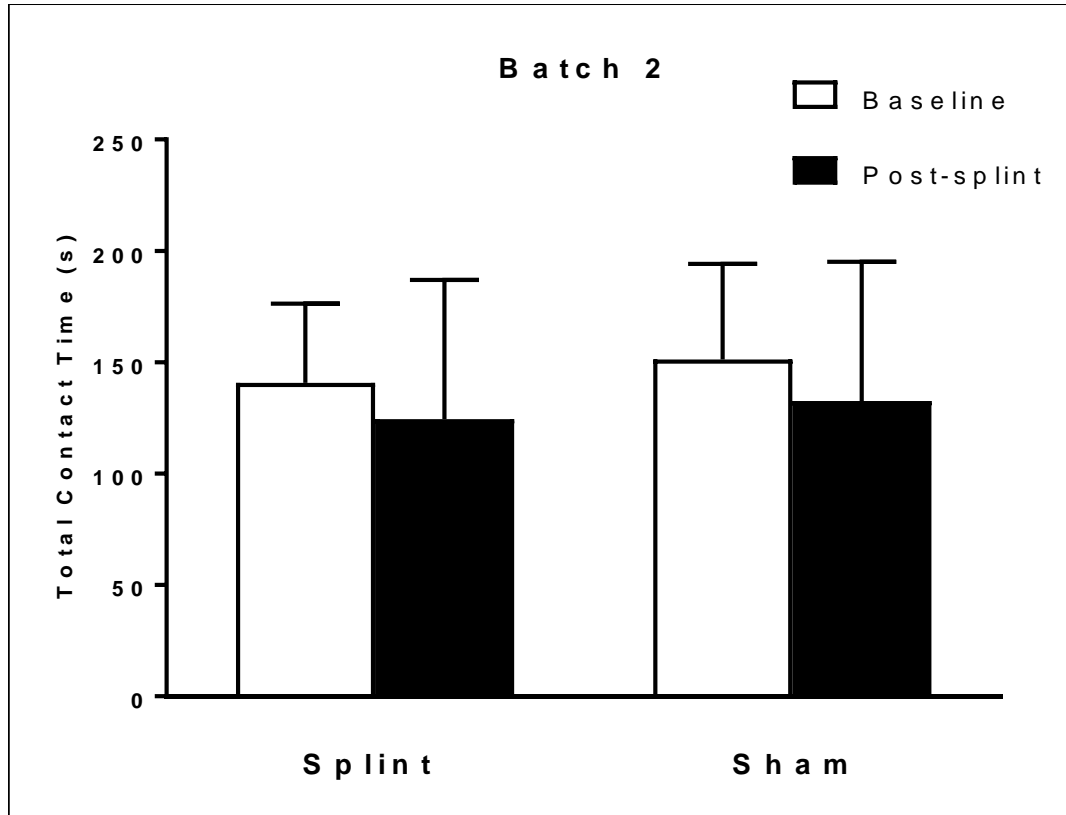


Figure 11 Averaged CT of all the rats in Batch 2

The CTs before and after the splint were compared in all the rats to evaluate the effect of splint placement on orofacial hypersensitivity. No significant difference was seen before and after splint placement in both groups ($\alpha=0.05$).

4.1.3 Male Rats Splinted for 4 Weeks

Batch 3 has 8 male rats, which are divided into two groups as in Batch 2. Even with 30% sucrose water, the baseline CTs were still low in comparison to Batch 2. With the concern of rats losing interest in the sucrose water, we added re-habitation sessions each week post-surgery.

The line charts show the CT changes of each rat in the splint group (Figure 12) and the Sham group (Figure 13). For the splint group, Rat 1 and 2 showed a decrease as early as the second day of the splint placement. Rat 3 has a sudden increase 2 days post-splint, followed by a gradual decline throughout the test. For Rat 4, there was a slow decrease in CT since the first day after splint placement. The results in the splint group might indicate the development of orofacial hypersensitivity. For the Sham group, even with the re-habitation, Rat 3 and 4 showed a slow decrease in the CT, while Rat 1 and 2 fluctuated heavily.

The averaged CT of the Sham group showed a slowly decrease trend. The splint group also showed a similar pattern to the Sham group despite some sudden increase in day 2 and 8 post-splint (Figure 14).

After averaging the CTs for each rat with respect to the two periods, only Rat 1 in the splint group and Rat 3 in the Sham group showed significant difference before and after the splint placement ($\alpha=0.05$, Figure 15), while no significant difference was seen after combining the CTs for all the rats in both group ($\alpha=0.05$, Figure 16). This might be due to the baseline CTs not being stable, and occasional fluctuation in the post-splint data, which increased the variation of the CTs.

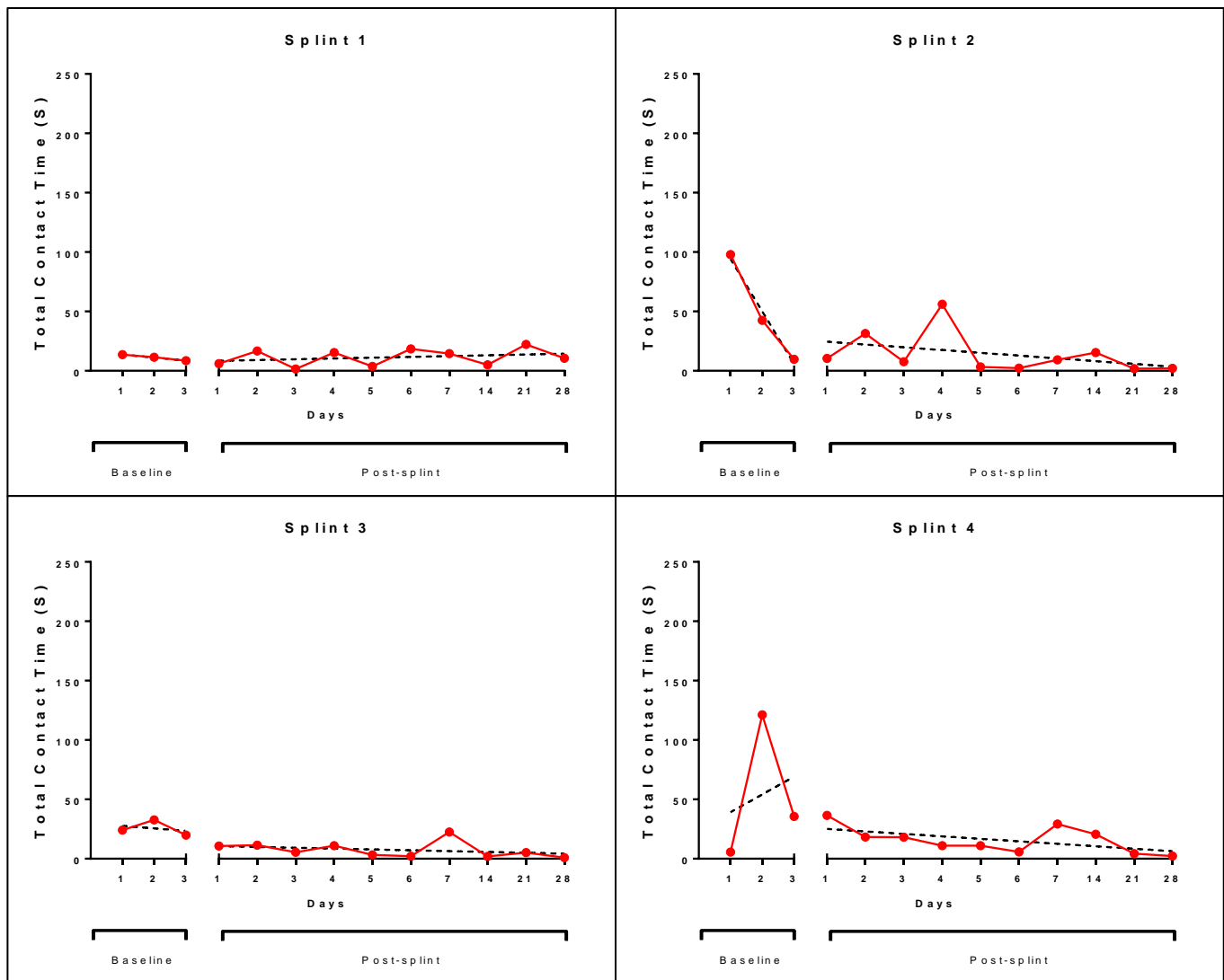


Figure 12 CT changes of each rat in the splint group in Batch 3

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. The CTs of Rat 1 and 2 decreased as early as the second day of the splint placement. Rat 3 showed a sudden increase in CT 2 days after splinting, followed by a gradual decrease throughout the rest of the test. The CT of Rat 4 decreased gradually since the first day after splint placement. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

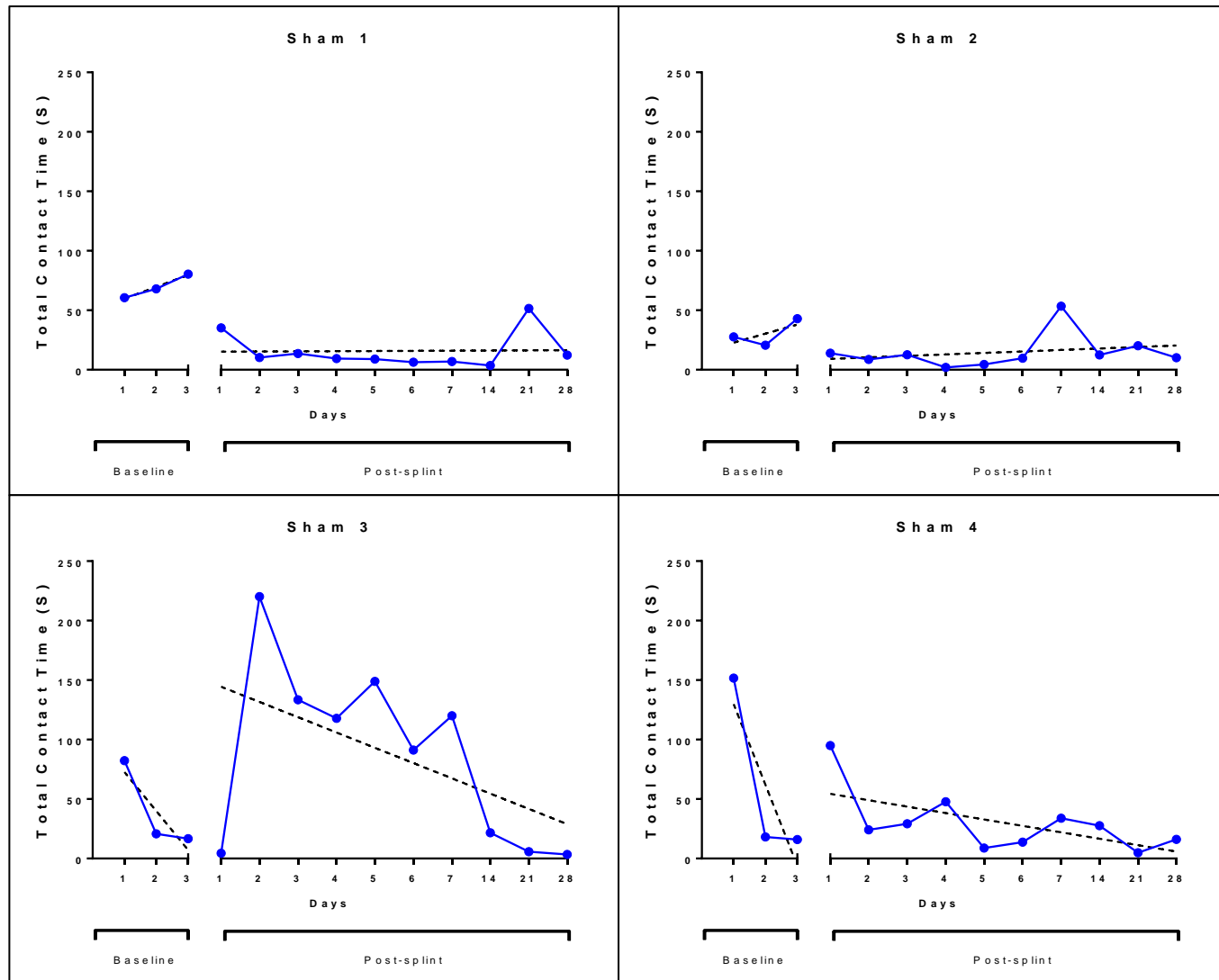


Figure 13 CT changes of each rat in the Sham group in Batch 3

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. Rat 1 and 2 fluctuated heavily, while Rat 3 and 4 showed a slow decrease in CT. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

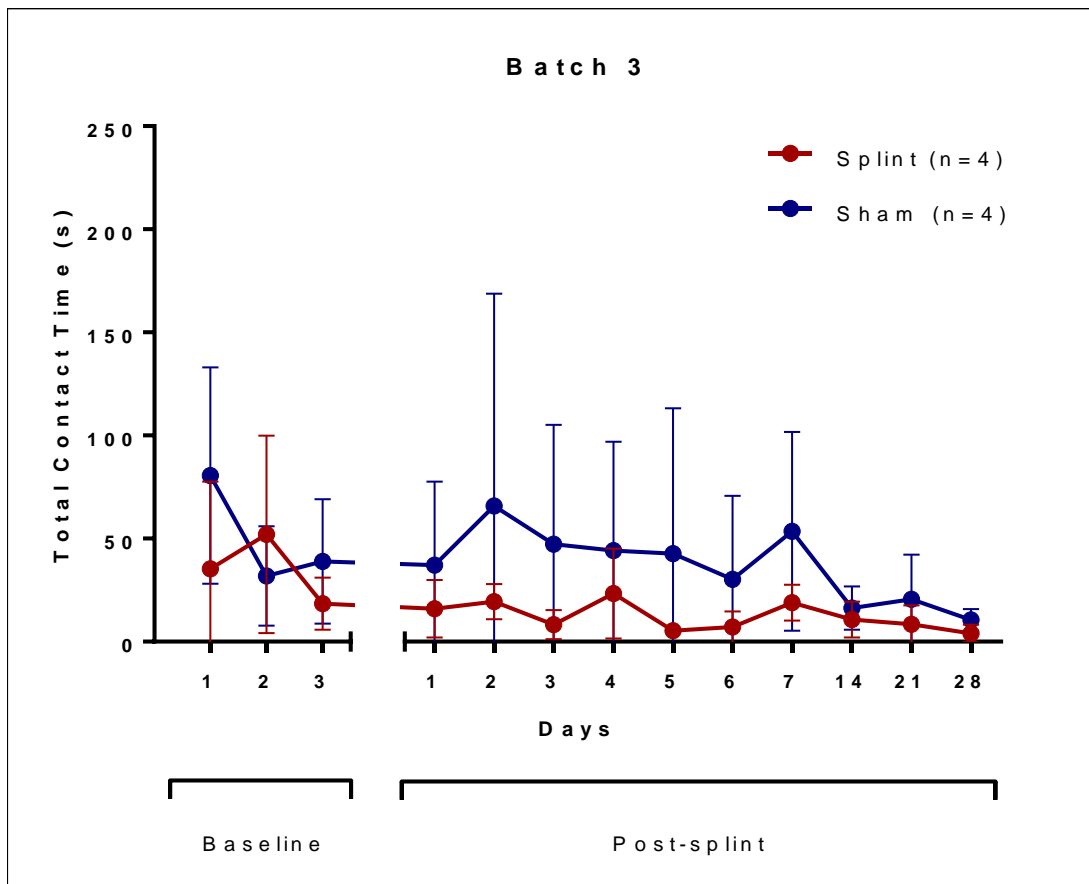


Figure 14 Averaged CT changes of all the rats in Batch 3

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. After averaging the CTs of all the 4 rats in the splint and Sham group, Sham group showed a slowly decrease trend, while the splint group also showed a similar pattern but with some sudden increase in day 2 and 8 post splint.

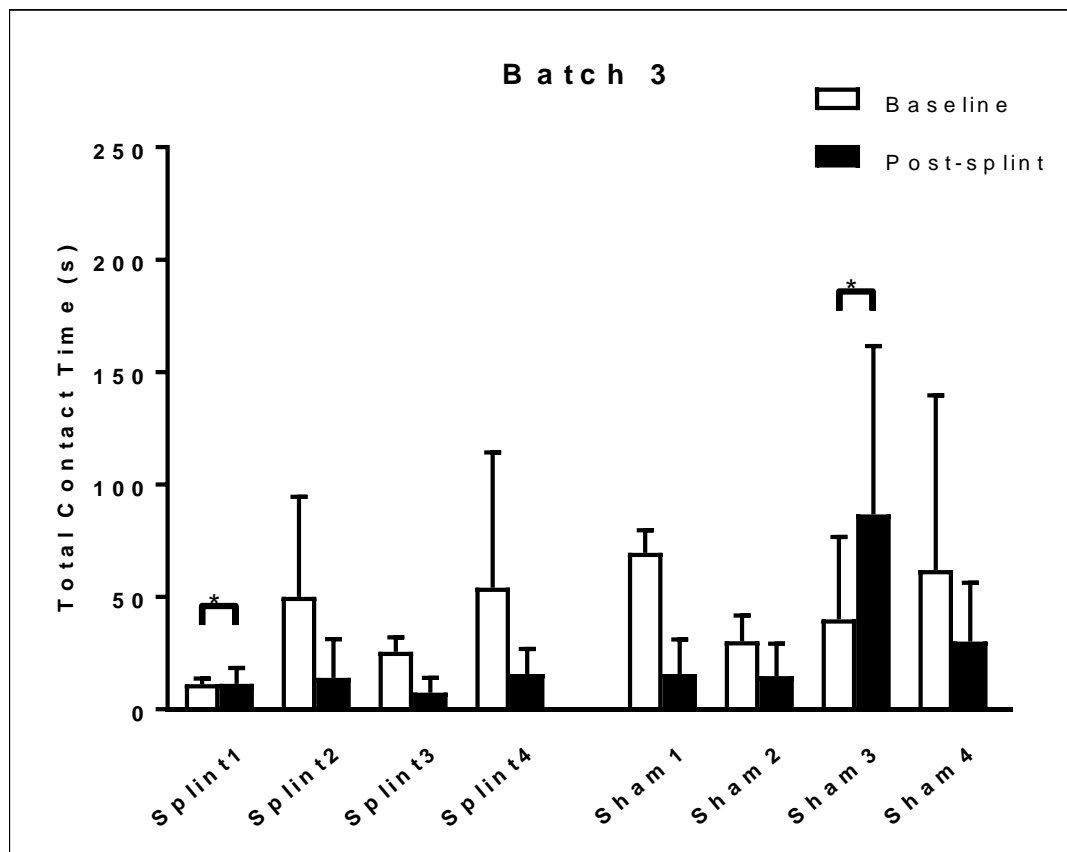


Figure 15 Averaged CT of each rat in Batch 3

The CTs before and after the splint were compared in each rat to evaluate the effect of splint placement on orofacial hypersensitivity. Rat 1 in the splint group and Rat 3 in the Sham group showed a significant difference. The averaged CTs for other rats, both in the splint and Sham group, did not show any significant difference. The asterisk indicates a statistical difference ($\alpha=0.05$).

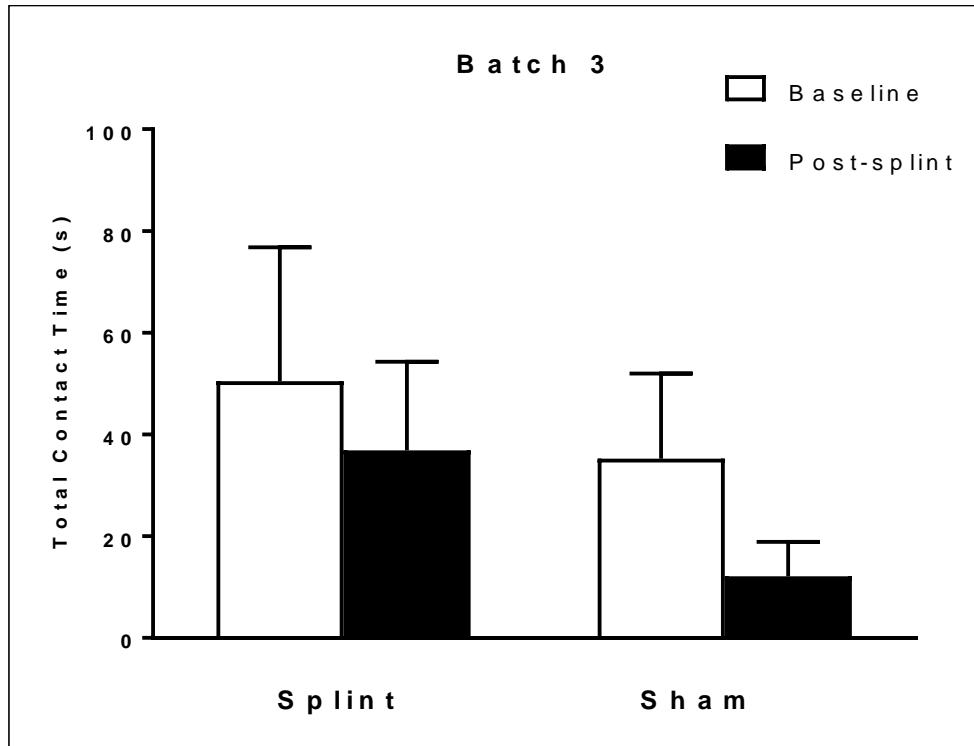


Figure 16 Averaged CT of all the rats in Batch 3

The CTs before and after the splint were compared in all the rats to evaluate the effect of splint placement on orofacial hypersensitivity. No significant difference was seen before and after splint placement in both groups ($\alpha=0.05$).

4.1.4 Female Rats Splinted for 6 Weeks

Batch 4 was handled by an experienced investigator, Jorge Pineda Farias, who was blinded to the splint and Sham groups. It has the same group setup as Batch 3 except that they were all female rats. In order to understand the influence of re-habitation on the rats' long-term interest in the sucrose water, the re-habitation was substituted with normal testing. The result showed that there is no influence of re-habitation on the rats' long-term interest in the sucrose water (data not shown). A prolonged test period (6 weeks) was employed to investigate the CT changes for an extended period.

The line charts show the CT changes of each rat in the splint group (Figure 17) and the Sham group (Figure 18). All the rats had high CT value before the splint placement. For the splint group, the CT of Rat 1 declined after the splint placement for the first week, followed by a gradual increase afterward. The CT of Rat 2 fluctuated for the first week and then soared to around 200s for the rest of the test. The CT of Rat 3 fluctuated but show an overall decreasing trend. For the Sham group, the CTs of all the 4 rats varied but remained relatively stable at around 200s.

The averaged CT of Sham group remained stable at around 200s, while the splint group declined for the first week and then return to almost the same level as the Sham group in the following weeks (Figure 19).

After averaging the CTs for each rat with respect to the two periods, Rat 2 in the splint group while Rat 1 and 3 in the Sham group showed significant difference before and after the splint placement ($\alpha=0.05$, Figure 20).

No significant difference was seen after combining the CTs for all the rats in the splint group, but the averaged CT for the Sham group increased significantly after splint placement ($\alpha=0.05$, Figure 21).

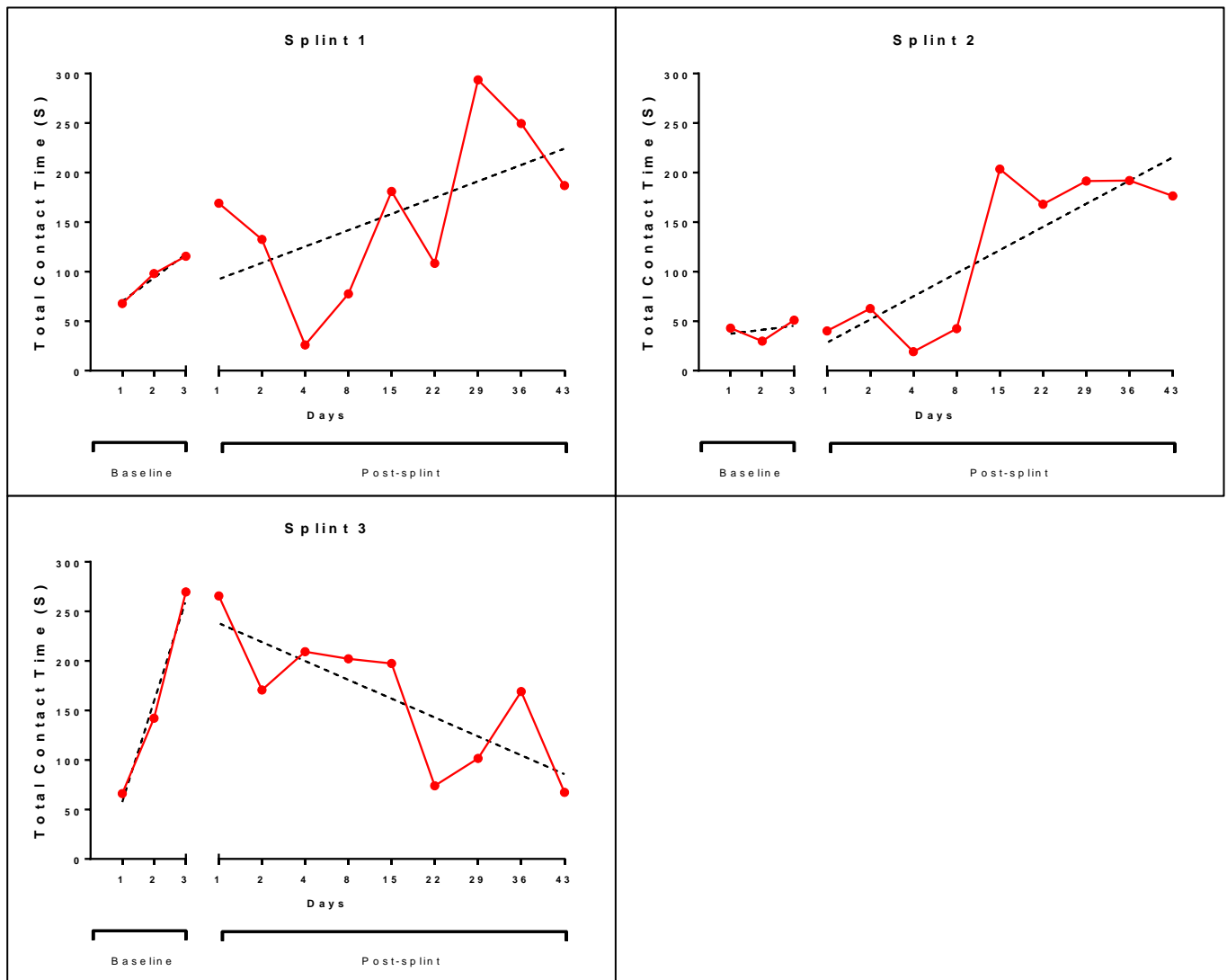


Figure 17 CT changes of each rat in the splint group in Batch 4

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. The CT of Rat 1 declined after the splint placement for the first week, followed by a gradual increase afterward. The CT of Rat 2 fluctuated for the first week and then soared to around 200s for the rest of the test. The CT of Rat 3 fluctuated but show an overall decreasing trend. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

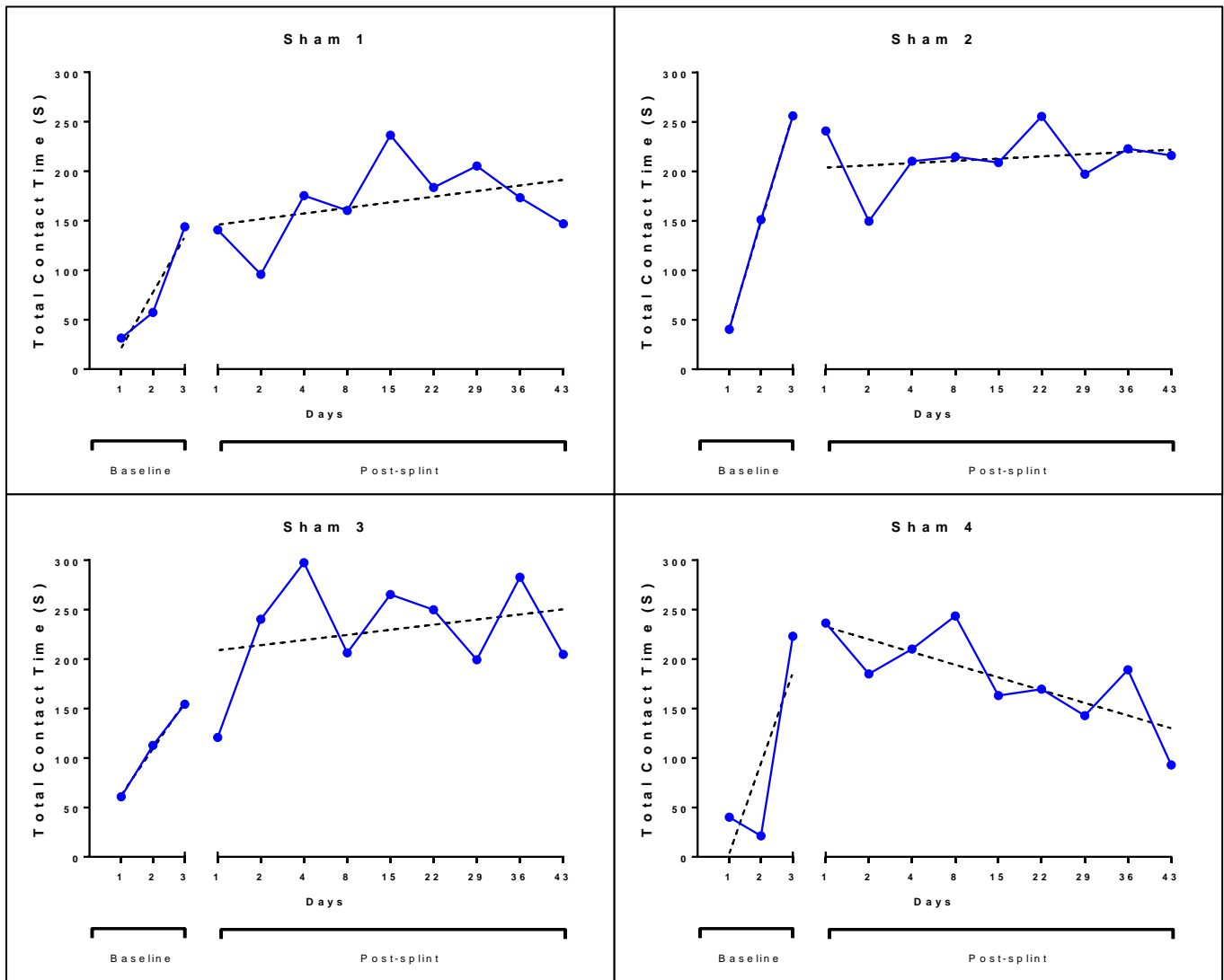


Figure 18 CT changes of each rat in the Sham group in Batch 4

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. The CTs of all the 4 rats fluctuated but remained relatively stable at around 200s. The dotted lines are the linear regression calculated from the data, which showed the trend of the CTs.

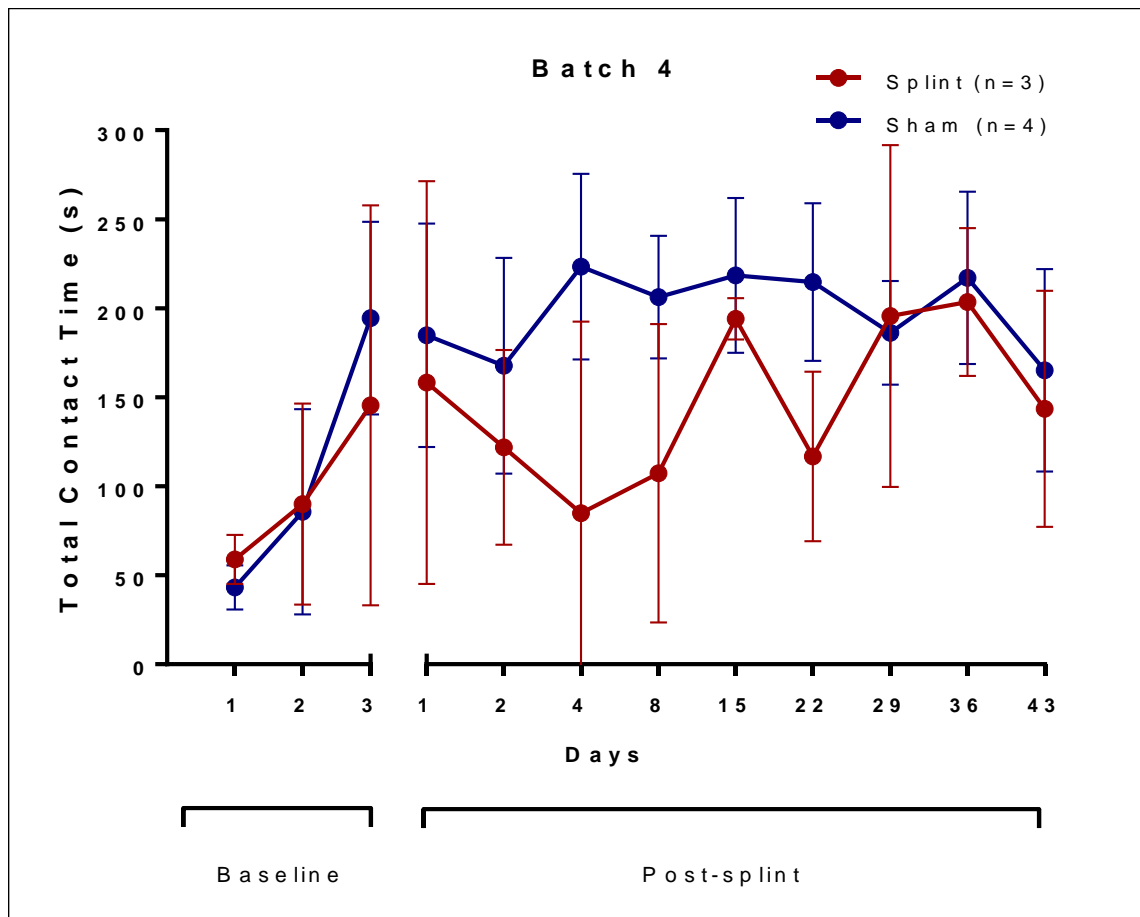


Figure 19 Averaged CT changes of all the rats in Batch 4

The gap in the x-axis indicates the time of splint placement. The numbers after the gap represent the corresponding time points after the splint placement. After averaging the CTs of all the 4 rats in the splint and Sham group, Sham group remained stable at around 200s, while the splint group declined for the first week and then return to almost the same level as the Sham group in the following weeks.

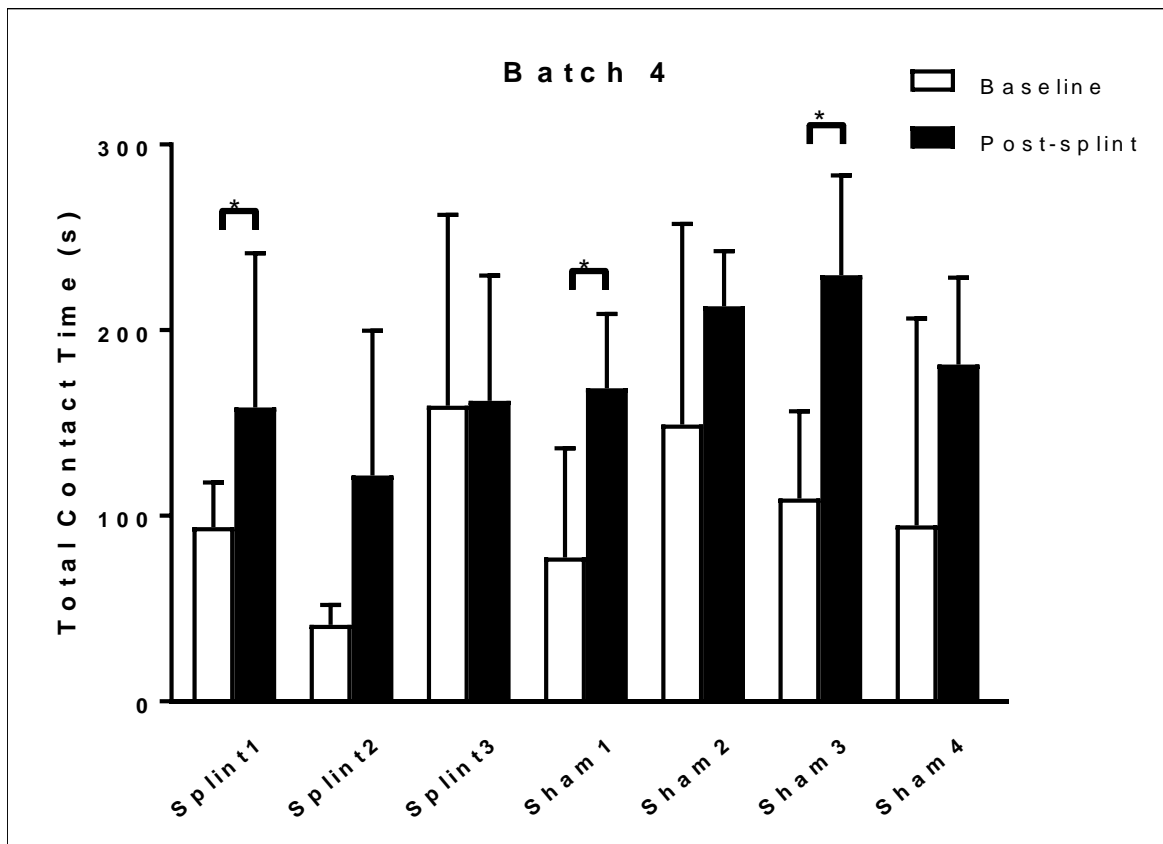


Figure 20 Averaged CT of each rat in Batch 4

The averaged CTs before and after the splint were compared in each rat to evaluate the effect of splint placement on orofacial hypersensitivity. Rat 2 in the splint group and Rat 1 and 3 in the Sham group showed a significant difference before and after the splint placement while other rats showed no statistical difference ($\alpha=0.05$).

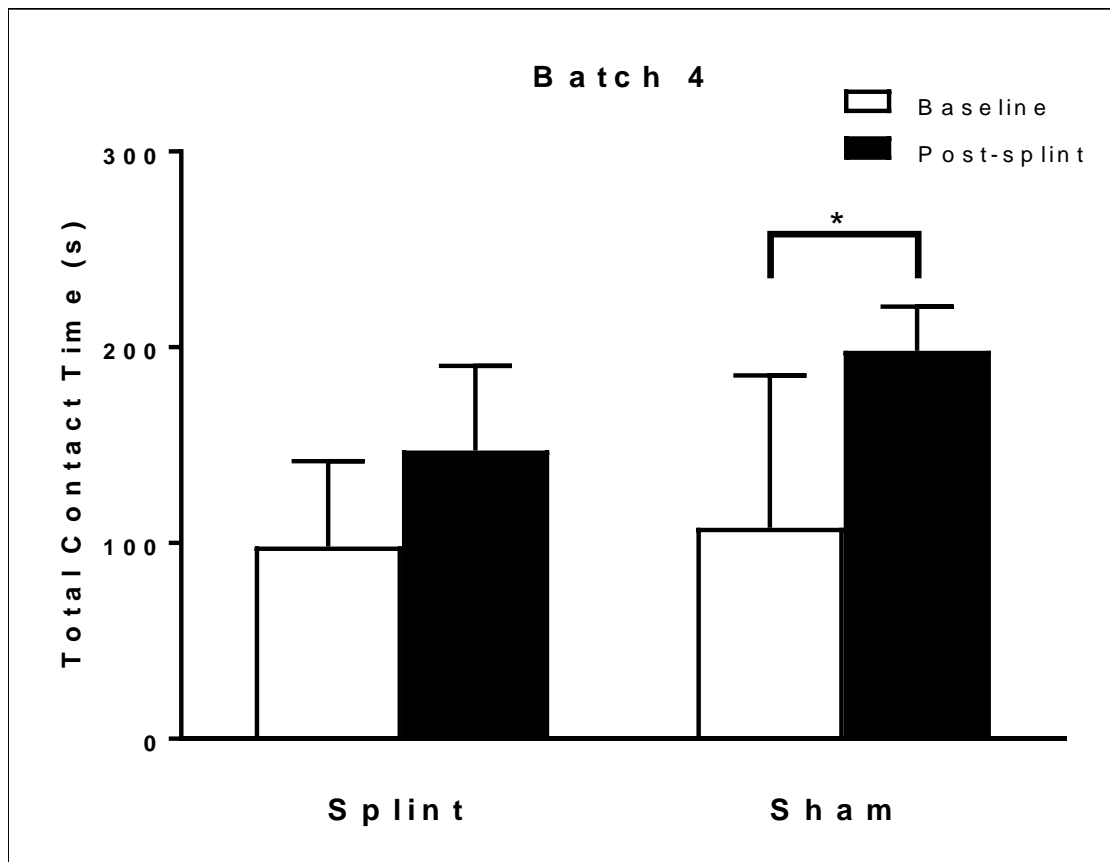


Figure 21 Averaged CT of all the rats in Batch 4

The CTs before and after the splint were compared in all the rats to evaluate the effect of splint placement on orofacial hypersensitivity. No significant difference was seen before and after the splint placement in the splint group, but the averaged CT for the Sham group increased significantly after splint placement. The asterisk indicates a statistical difference ($\alpha=0.05$).

4.1.5 Weight Change

The weight of each rat in Batch 3 (Figure 22) and Batch 4 (Figure 23) was monitored and recorded throughout the pre-splint and post-splint period. Although there were some variations between individuals, all the rats showed a steady increasing trend in weight. There was no apparent difference in weight gaining between splint and Sham group.

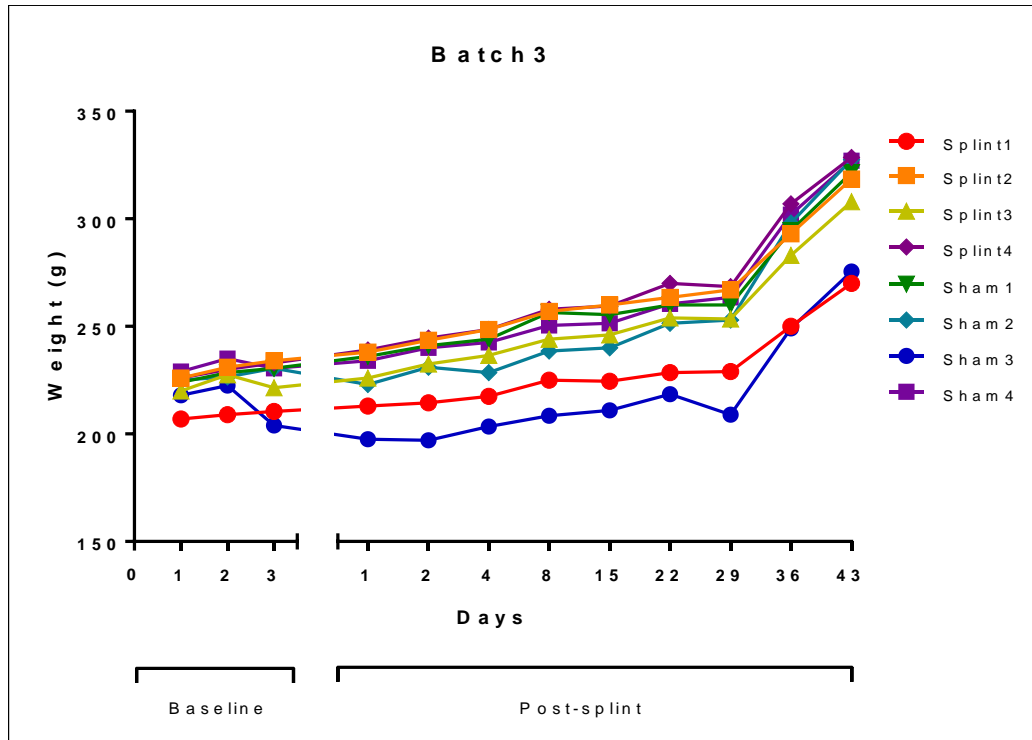


Figure 22 Weight change of Batch 3

The weight of each rat in Batch 3 was monitored throughout the pre-splint and post-splint period. Although there were some variations between individuals, all the rats showed a steady increasing trend in weight. There was no apparent difference in weight gaining between splint and Sham group.

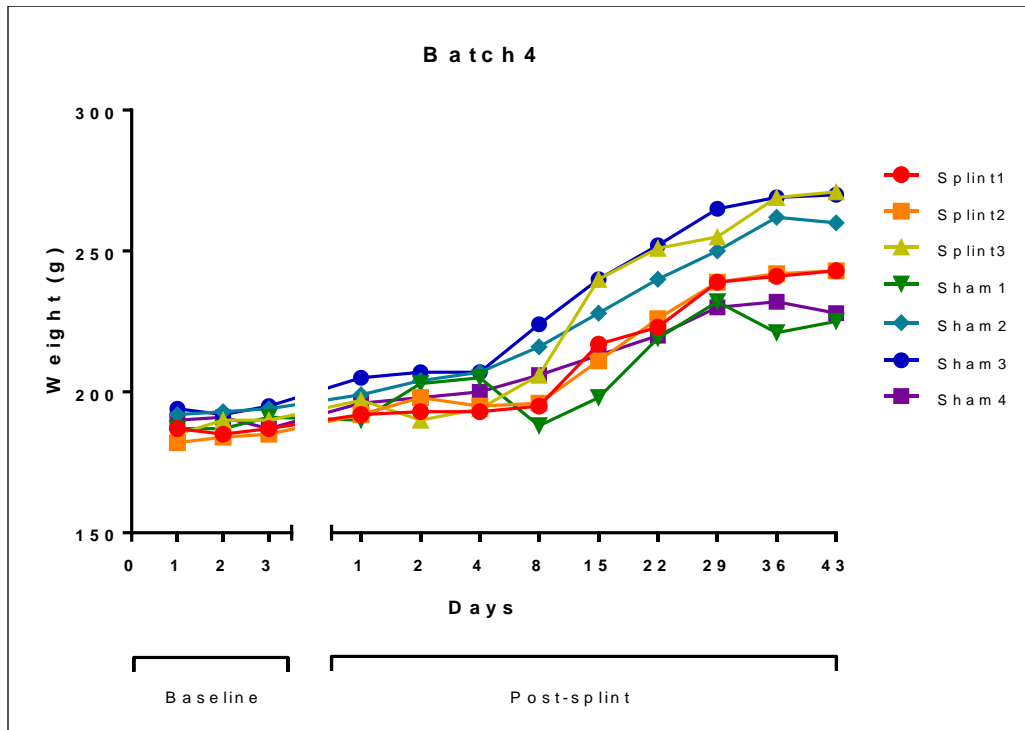


Figure 23 Weight change of Batch 4

The weight of each rat in Batch 4 was monitored throughout the pre-splint and post-splint period. Although there were some variations between individuals, all the rats showed a steady increasing trend in weight. There was no apparent difference in weight gaining between splint and Sham group.

5.0 Discussion

5.1 Animal TMJ degeneration model

A number of TMJ degeneration models involving interocclusal bite planes have been developed (Sergl and Farmland 1975, Shaw and Molyneux 1993, Chaves, Munerato et al. 2002, Henderson, Lowe et al. 2015). By using different TMJ degeneration models, researchers are trying to study the impact of altered load on the TMJ and the surrounding soft tissue.

One type of TMJ degeneration models is to induce occlusal trauma, which involves a bite-raising splint inserted to bare and/or shift the burden of mastication (Sergl and Farmland 1975). The splint to be inserted includes crown, resin, or metal. The location of the insertion could be either molar (one side or both side) or incisors (Chaves, Munerato et al. 2002, Henderson, Lowe et al. 2015).

Another type of TMJ degeneration model tries to decrease the load of the TMJ by trimming down the incisors or administering soft food diet (Hinton and Carlson 1986, Hinton 1988). Such changes are believed to decrease the loading applied on the mandibular condyle, causing structural and content changes in the fibrocartilage layer.

It is well established that altered TMJ masticatory loading in mammals can lead to degenerative TMJ disorder (Wadhwa and Kapila 2008), resulting in the thickening of the condylar fibrocartilage (Bouvier 1988, Kiliaridis, Thilander et al. 1999, Ravosa, Kunwar et al. 2007), reduction of extracellular matrix protein expression in the cartilage (Pirttiniemi, Kantomaa et al. 1996), and chondrocyte proliferation (Hinton 1988, Pirttiniemi, Kantomaa et al. 2004, Sato, Uneno et al. 2006).

In the previous rabbit TMD model our lab developed using unilateral molar splint, we found that the condylar fibrocartilage layers from the splinted rabbits underwent significant remodeling characterized by a change in the distribution of GAG and collagen II, accompanied by a loss of defined cell layers. Besides, the stiffness of the condylar fibrocartilage was significantly higher while the DNA content was significantly lower than the control group (Henderson, Lowe et al. 2015).

In this study, we used unilateral resin splint on the molar for 3 reasons: 1. It has the potential to induce altered load on different sides; 2. It is clinically relevant; 3. It is easy to insert, and it is reversible so that it can be removed at any time. Furthermore, the use of splints causes damage to the joint surface, this allows for the elucidation of the relationship between joint degeneration and pain, without the confounding variable of inflammation.

5.2 Pain/Hypersensitivity Assessment

The evaluation of orofacial pain/hypersensitivity in animals is challenging, because it cannot be measured directly. Thus, several indirect pain assessment methods have been developed to quantify and evaluate the pain-like behavior. These pain assessment methods can be categorized into stimulus-evoked tests and stimulus-independent tests (Deuis, Dvorakova et al. 2017).

Stimulus-evoked tests are the most commonly used methods to test the pain/hypersensitivity for rodents. It involves the use of nociceptive stimulus, which are usually innocuous, but rats will become aversive if there is allodynia or hyperalgesia in the testing area. Von Frey Hair (VFH), designed by Maximilian von Frey in 1896, is commonly used as the stimulus-evoked test for nociception, in which filaments of different forces are applied

perpendicularly to the skin surface to determine the threshold force that evokes flinch (Vos, Strassman et al. 1994, Bradman, Ferrini et al. 2015). Orofacial Pain Assay, or Orofacial Operant Test, is another widely used stimulus-evoked test for nociception (Nolan, Hester et al. 2011, Krzyzanowska and Avendano 2012, Ramirez, Queeney et al. 2015). It has a hole through which the rat can put their head and get access to the reward solution. When getting the reward, its face will make contact with either mechanical (wire filaments) or thermal (hot or cold) stimulus on the hole. Such nociception stimuli will have no effect on normal rats but can induce discomfort or even pain on the face if the rat has allodynia or hyperalgesia, resulting in the reduction of the Contact Time with the reward solution and the number of licks. Nolan et al. used mechanical stimulus on the rats. The ratio of total Contact Time to the number of licks decreased significantly after the bilateral application of capsaicin to the face of the rats, which can be neutralized by the injection of morphine (Nolan, Hester et al. 2011). Thermal stimulus was also used in some other studies. Neubert et al. investigated the effect of hot stimulus on 6 outcome measures, reward intake, facial contact duration, licking contact events, facial contact event, reward/attempts, and facial duration/contact (Neubert, Widmer et al. 2005). 5 different temperatures were used (37.7 °C, 41.7 °C, 45.5 °C, 52.5 °C, and 57.2 °C). The result showed that as the temperature increased, all the outcome measures decreased except facial contact events. Another study investigated the effect of cold stimulus on reward intake, facial contact events, licks/facial contact, and duration/facial contact. The test was conducted with 5 different temperatures 37 °C, 24 °C, 10 °C, 2 °C, and -4 °C. The result indicated that low temperature tends to yield low intake, low licks/facial contact, low duration/facial contact, but high facial contact events (Rossi, Vierck et al. 2006).

Meal pattern analysis, which monitors the food intake pattern and food size preference, is used as nociceptive-independent test to assess pain (Varma, Chai et al. 1999, Kerins, Carlson et al.

2005, Kramer, Kerins et al. 2010). After injecting the Complete Freund's Adjuvant (CFA), a chemical that cause local inflammation, into the TMJ of rats, the meal pattern was significantly altered, characterized by extended meal duration and decreased number of meals with the same meal size(Harper, Kerins et al. 2000). These changes can be reversed by the administration of ibuprofen (IBU), an anti-inflammatory drug (Kerins, Carlson et al. 2003). The results demonstrate that meal pattern analysis can be used as a non-invasive behavior test for TMJ inflammation/pain. Beside meal pattern analysis, other nociceptive-independent methods include observational test by using grimace scale (Langford, Bailey et al. 2010, Sotocinal, Sorge et al. 2011) or face wash strokes (Vos, Strassman et al. 1994).

This study used the orofacial operant test because: (1) this is an investigator-independent system; (2) the test involves the movement of the jaw during the intake of the sucrose water, which makes it more clinically relevant to TMD.

5.3 Statistical Analysis

Doing statistical analysis by comparing the averaged CTs might not be the best method for this specific experiment, because the standard deviation could be very high due to: (1) the CTs fluctuated a lot before the splint placement and even in the early stage after the splint was put on; (2) most of the rats only showed the trend of decreasing CT with occasional variance during the post-splint period; (3) the CT difference between rats was huge even within the same day. For the reasons mentioned above, student t-test might not be a good choice for the statistical analysis before we figure out a way to eliminate the non-linear data. Alternatively, we could perform non-parametric data analyses.

5.4 Other Variables

By monitoring the weight, we were trying to assess the effect of hunger, caused by not being able to consume hard food after splint placement, on the CTs. The results in both Batch 3 and 4 showed that there is no noticeable difference in weight between the two groups before and after the splint placement, so it can be concluded that the splint placement will not pose difficulty in food intake and affect the CTs.

The idea of switching male and female rats was to investigate the influence of gender difference on the orofacial hypersensitivity. However, since the apparatus setup is not consistent between batches, and baseline CTs of Batch 1 and 3 were not high enough, we could not make the comparison between male and female rats.

6.0 Conclusion

Due to the change of experimental setup, the result from each batch is not comparable to others. In addition, the baseline CTs of Batch 1 and 3 is low, making the post-splint data not reliable. Thus, no conclusion can be drawn from the current study. However, in the future studies, more rats will be used, and we will adopt better data analysis methods, for example, Kruskal Wallis test, chi-square test, or two-way ANOVA. We will also introduce other behavior tests to assess the emergence of orofacial pain caused by the sudden change of occlusion, such as meal pattern analysis, which can indicate TMJ pain when an increased meal duration and decreased number of meals are seen.

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